Host-Feeding Pattern of *Culex theileri* (Diptera: Culicidae), Potential Vector of *Dirofilaria immitis* in the Canary Islands, Spain

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ABSTRACT To identify the host range of potential vectors of *Dirofilaria immitis* Leidy, the causal agent of canine dirofilariasis, we studied the bloodmeal origin of mosquitoes trapped on two of the Canary Islands, Gran Canaria and Tenerife, where this disease is considered hyperendemic. On Gran Canaria, mosquitoes were captured using Centers for Disease Control and Prevention (CDC) traps (outdoors) and resting in a bathroom (indoors). Only CDC traps were used to capture mosquitoes in Tenerife. The species captured in decreasing order of abundance were *Culex theileri* Theobald, *Culex pipiens* L., *Culiseta longiareolata* Macquart, *Anopheles atroparvus* van Thiel, and *Anopheles cinereus* Theobald. The origins of bloodmeals were identified for 121 *Cx. theileri* and 4 *Cx. pipiens* after amplification and sequencing of a fragment of the vertebrate cytochrome oxidase I (COI) gene. *Cx. theileri* fed on goats, sheep, dogs, cattle, cats, humans, and chickens, and *Cx. pipiens* fed on goats and chickens. A lower success of bloodmeal identification was obtained in mosquitoes captured resting indoors than outdoors in CDC traps, probably because of a longer time period between feeding and capture. Although most *Cx. theileri* fed on ruminants, this species also fed on different mammal species susceptible to dirofiliarasis, including humans, suggesting it could play a role on parasite transmission.

KEY WORDS Dirofilariasis, dog, host-parasite interaction, human, mosquito

The dirofilariasis caused by *Dirofilaria immitis* Leidy is a cosmopolitan disease affecting domestic and wild mammals and humans (McCall et al. 2008, Simón et al. 2009). In the Canary Islands, dogs, cats, and humans show high seroprevalence for *D. immitis* (Montoya et al. 1998, 2006; Montoya–Alonso et al. 2011a,b). Most evidence indicates that *Culex theileri* Theobald is probably the main vector of *D. immitis* on these islands (Morchón et al. 2011, Santa–Ana et al. 2006), although other *Culex* species and mosquitoes belonging to other genera also have been incriminated in the transmission (Shaw and Day 2005, Cancrini et al. 2007, Morchón et al. 2012, Latrofa et al. 2012). In endemic regions for *D. immitis*, as in the case of the Canary Islands, humans and other potential hosts (e.g., domestic animals) are at risk of being bitten by infected mosquitoes and potentially suffer parasite infection. Despite this veterinary and public health problem, to our knowledge, there have been no studies on the blood feeding behavior of these potential vectors in the Canary Islands. In fact, there have been few studies on the host-feeding patterns of *Culex* mosquitoes in Europe (Balenghien et al. 2006; Muñoz et al. 2011, 2012; Roiz et al. 2012a,b,; Osório et al. 2012; Ventim et al. 2012), especially for *Cx. theileri* (Muñoz et al. 2011, 2012; Osório et al. 2012).

The identification of bloodmeal origins is essential to determine the vector host range and pathogen transmission networks. Traditionally, host identification was done using serological techniques (Braverman et al. 1971, Blackwell et al. 1994, Mwangangi et al. 2003), but recently, these studies have progressively incorporated sequencing techniques based on the amplification of the bloodmeal host's DNA (Kent 2009, Gómez–Díaz and Figuerola 2010). Thus, the purpose of the current study was to identify the bloodmeal sources of mosquitoes captured on two of the Canary Islands, Gran Canaria and Tenerife, using the recently developed molecular approach described by Alcaide et al. (2009). This method is based on the Barcoding of Life program that aims to provide a reference library of cytochrome oxidase I (COI) sequences of all vertebrates and other organisms on earth (Hebert et al. 2003, Hajibabaei et al. 2007).

Materials and Methods

Study Area. Mosquitoes were captured from September to December 2010 during a study on bluetongue vectors (Martínez–de la Puente et al. 2012). Centers for Disease Control and Prevention (CDC)-

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Table 1. Female mosquitoes captured in this study

	Gran Canaria (CDC)		Gran Canaria (resting)		Tenerife (CDC)	
	Unfed	Blood-fed	Unfed	Blood-fed	Unfed	Blood-fed
Cx. theileri	186	35	131	201	55	23
Cx. pipiens	13	4	13	15	0	0
Culex spp.	0	0	8	0	30	0
Cs. longiareolata	2	0	0	0	0	0
An. atroparvus	0	0	0	0	1	0
An. cinereus	0	0	1	0	0	0

Mosquitoes were captured using CDC traps in Gran Canaria and Tenerife and resting in the bathroom in Gran Canaria.

type downdraft miniature suction traps (model 1212; J.W.Hock, Gainesville, FL) operating with a 4-W black light (ultraviolet) bulb were used to capture mosquitoes at two farms, one on Gran Canaria (the farm of the Universidad de Las Palmas de Gran Canaria, 28°8′21 N, 15°30′24 W) and one on Tenerife (the farm of the Instituto Canario de Investigaciones Agrarias, 28°31′24 N, 16°22′19 W). Traps were operated from sunset to sunrise with an irregular periodicity and were hung at 2.2 m above the ground to keep farm animals away from the traps. Traps were located very close to animal corrals containing goats and/or sheep and, in the case of Gran Canaria, ≈10 m from kennels containing dogs. Additional potential host species including humans (e.g., farm workers and students, the last for the case of Gran Canaria), other mammals and different bird species were observed near trap locations. In addition, during September and October 2011, mosquitoes were captured by hand, during daylight, resting in the bathroom of the farm of Gran Canaria, which was ≈2 m from corrals containing animals. This was the only location where resting mosquitoes were observed at this farm.

Mosquitoes were preserved at −80°C until identification. In the laboratories of the Servicio de Control de Mosquitos (Diputación Provincial de Huelva), mosquitoes were enumerated to species using available morphological keys (Brunhes et al. 2000, Schaffner et al. 2001), and females containing a bloodmeal were individually stored until analyses of bloodmeal origin.

Bloodmeal Identification. The abdomen of each blood-fed mosquito was removed using sterile pipet tips, placed into 75 μl of lysis solution (25 mMNaOH, 0.2 mM EDTA), crushed and incubated at 95°C for 30 min. After incubation, the solution was cooled on ice for 5 min and then 75 μl of neutralization solution (40 mM Tris-HCl) was added according to the HotSHOT procedure (Truett et al. 2000). At least two negative DNA extraction controls (i.e., absence of blood) were included per plate. Abdomens were processed on 96-thermowell plates and DNA extracts were stored at −20°C until polymerase chain reaction (PCR) amplification. Bloodmeal origin was determined using the protocol described by Alcaide et al. (2009) to amplify a 758 bp fragment of the vertebrate COI gene. Sequencing reactions were performed according to BigDye 1.1 technology (Applied Biosystems, Carlsbad, CA) and labeled DNA fragments were resolved through an ABI 3130xl automated sequencer (Applied Biosystems). Sequences were edited using Sequencher v4.9 software (Gene Codes, 1991–2009, Ann Arbor, MI). Host species identification was done by comparison with sequences deposited in GenBank DNA sequence database (National Center for Biotechnology Information Blast) or the Barcode of Life Data Systems (BOLD Systems platform). Agreement of ≥98% with known species sequences was required for host identification.

Results

In total, 774 mosquitoes (56 males and 718 females) were captured (Table 1). The most abundant mosquito species was *Cx. theileri* ($n = 672$), followed by *Culex pipiens* L. ($n = 57$), *Culiseta longiareolata* Macquart ($n = 5$), *Anopheles atroparvus* van Thiel ($n = 1$), and *Anopheles cinereus* Theobald ($n = 1$). Because of the loss of some anatomical structures, 38 mosquitoes were only identified to *Culex*. Overall, 259 *Cx. theileri* and 19 *Cx. pipiens* females contained blood in their abdomen, but successful amplification and identification of the bloodmeal source was done only for 121 *Cx. theileri* and four *Cx. pipiens*. On Gran Canaria, *Cx. theileri* fed blood on goats *Capra hircus* ($n = 79$), dogs *Canis lupus familiaris* ($n = 11$), sheep *Ovis aries* ($n = 10$), cat *Felis catus* ($n = 1$), cattle *Bos taurus* ($n = 1$), chicken *Gallus gallus* ($n = 1$), and human *Homo sapiens* ($n = 1$). In addition, one *Cx. theileri* from Gran Canaria showed clear double peaks at different positions of the sequencing electropherogram suggesting that it fed blood on two different hosts. By comparing this sequence with those from the other bloodmeals obtained in this study, the sequence with double peaks was identified as goat and dog. The seven hosts species were identified from *Cx. theileri* mosquitoes captured resting in the bathroom, whereas only goats and sheep were the hosts of *Cx. theileri* mosquitoes captured using CDC traps. Moreover, goats were the bloodmeal sources of 16 mosquitoes from Tenerife. Bloodmeals identified from *Cx. pipiens* corresponded to goats ($n = 3$) and chicken ($n = 1$).

On Gran Canaria, significantly greater success was obtained in the identification of the bloodmeal sources in mosquitoes trapped using CDC traps (25 of 39; 64.10%) than resting in the bathroom (84 of 216; 38.89%) ($\chi^2 = 8.58$; $P = 0.003$). A similar success in the identification of bloodmeal sources was obtained from mosquitoes captured using CDC-traps in Tenerife (16

of 23; 69.57%) and Gran Canaria (Yates corrected $\chi^2 = 0.03$; $P = 0.87$).

Discussion

Cx. theileri is mainly considered a mammophilic mosquito but also feeds on birds (Jupp et al. 1980, Alcaide et al. 2009, Muñoz et al. 2012, Osório et al. 2012). In different wetlands in south–western Spain, the proportion bloodmeals of mammal origin ranged from 77.8 to 91.2%, with very low prevalence of bloodmeals from humans (Muñoz et al. 2012). In agreement with these results, we found that *Cx. theileri* fed extensively on ruminants (goats, sheep, and cattle) in addition to dogs, cats, humans, and chickens. Previously, dogs, cats, and humans had been identified as hosts in a study of 11 bloodfed *Cx. theileri* from Portugal using a molecular approach (Osório et al. 2012). Overall, these results should be considered of interest in the transmission of *D. immitis*, because *Cx. theileri* is a potential vector of *D. immitis* (Santa–Ana et al. 2006, Morchón et al. 2011). Although *Cx. pipiens* could be also involved in the transmission of this pathogen (Shaw and Day 2005, Morchón et al. 2012), the low number of bloodmeals identified in this study provided minimal information. According to previous studies, the percentage of human derived feeds in *Culex* mosquitoes varies widely ranging, for example, in *Cx pipiens* from 0 to 35.7% (Gómez–Díaz and Figuerola 2010, Muñoz et al. 2011). This variation may be affected by different factors including host preference and the density of humans and alternative hosts in the area. This fact may potentially affect the transmission of *D. immitis* because, although humans are susceptible to parasite infection, people are considered dead end hosts for the parasite. Moreover, although *Cx. theileri* can also transmit West Nile virus (WNV; Hubálek and Halouzka 1999), the high prevalence of mammal bloodmeals indicated that the risk of WNV amplification and tangential transmission by this mosquito would be minimal.

Previous studies have reported high seroprevalence to *D. immitis* in dogs, cats, and humans in the Canary Islands (Montoya–Alonso et al. 2011a,b). According to our results, in presence of other potential hosts of *Cx. theileri*, a relatively low percentage of mosquitoes fed on dogs, the definitive host of *D. immitis*. These results could be because of the higher abundance of goats in reference to other potential hosts in the area, although unfortunately, we did not collect exhaustive data on host abundance to test this possibility. Curiously, we found a single bloodmeal from humans, although humans were present in the area and most blood-fed mosquitoes were trapped in the bathroom, a place exclusively used by people. Most likely *Cx. theileri* were not feeding in the bathroom but were using this as a resting site. This was indicated by the greater number of host species that were identified from mosquitoes captured resting in the bathroom than in those captured using CDC traps. It is possible that the method of insect capture biased bloodmeal samples, with blood-fed females from CDC traps feeding on a reduced subset of available host species (Thiemann and Reisen 2012). However, the most parsimonious explanation is that the observed differences were because of the great number of blood-fed females captured resting in the bathroom than in outdoors traps.

Finally, although most of the blood-fed mosquitoes were captured in the bathroom, a significantly lower proportion of the hosts of these bloodmeals were identified. This is probably because of a greater degradation of the host's DNA because of an increase in time between feeding and insect capture in comparison to those mosquitoes captured using CDC traps. It is known that the efficacy of identification of bloodmeal sources varies according to the stage of digestion of the host's DNA in the abdomen of the mosquito (Mukabana et al. 2002, Oshaghi et al. 2006, Haouas et al. 2007). In *Anopheles* mosquitoes the proportion of successfully identified bloodmeals drops below 50% at 15 h after ingestion (Mukabana et al. 2002). In a previous study, a considerable number of bloodmeals from mosquitoes captured indoors, and probably resting for an extended time period, could not be identified by using an assay based on the amplification of a fragment of 132- to 680-bp of host's DNA (Fornadel and Norris 2008). However, Thiemann et al. (2011) were able to identify *Culex* bloodmeals up to 3 d after blood feeding using COI. The method of extraction is also likely to affect amplification success given the degraded nature of many of the DNA samples analyzed. HotSHOT is a quick and cheap method for DNA extraction (Alcaide et al. 2009), but use of other protocols or commercial kits for degraded DNA extraction may increase amplification success.

The main conclusion of our study is that in the Canary Islands, the potential *Dirofilaria* vector, *Cx. theileri*, fed on blood from different ruminant species and on the potential hosts *D. immitis* (including dogs, cats, and humans). Moreover, we found support to the fact that bloodmeal degradation probably led to reduced polymerase chain reaction amplification and sequencing of a 758 bp fragment of the host's COI gene.

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References Cited

Alcaide, M., C. Rico, S. Ruiz, R. Soriguer, J. Muñoz, and J. Figuerola. 2009. Disentangling vector-borne transmission networks: a universal DNA barcoding method to identify vertebrate hosts from arthropod bloodmeals. PLoS ONE 4: e7092.

Balenghien, T., F. Fouque, P. Sabatier, and D. J. Bicout. 2006. Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. J. Med. Entomol. 43: 936–946.

Blackwell, A., A.J.M. Mordue (Luntz), and W. Mordue. 1994. Identification of bloodmeals of the Scottish biting midge, *Culicoides impunctatus*, by indirect enzyme-linked immunosorbentassay (ELISA). Med. Vet. Entomol. 8: 20–24.

Braverman, Y., P.F.L. Boreham, and R. Galum. 1971. The origin of blood meals of female *Culicoides pallidipennis* trapped in a sheepfold in Israel. J. Med. Entomol. 8: 379–381.

Brunhes, J., A. Rhaim, B. Geoffroy, G. Angel, and J.-P. Hervy. 2000. Les moustiquesdeel'Afriquemediterranéenne. CD ROM PC.IRD Editions.

Cancrini, G., P. Scaramozzino, S. Gabrielli, M. Di Paolo, L. Toma, and R. Romi. 2007. *Aedes albopictus* and *Culex pipiens* implicated as natural vectors of *Dirofilaria repens* in Central Italy. J. Med. Entomol. 44: 1064–1066.

Fornadel, C. M., and D. E. Norris. 2008. Increased endophily by the malaria vector *Anopheles arabiensis* in Southern Zambia and identification of digested blood meals. Am. J. Trop. Med. Hyg. 79: 876–880.

Gómez–Díaz, E., and J. Figuerola. 2010. New perspectives in tracing vector-borne interaction networks. Trends Parasitol. 26: 470–447.

Hajibabaei, M., G.A.C. Singer, P.D.N. Hebert, and D. A. Hickey. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 23: 167–172.

Haouas, N., B. Pesson, R. Boudabous, J. P. Dedet, H. Babba, and C. Ravel. 2007. Development of a molecular tool for the identification of *Leishmania* reservoir hosts by blood meal analysis in the insect vectors. Am. J. Trop. Med. Hyg. 77: 1054–1059.

Hebert, P.D.N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B. 270: 313–321.

Hubálek, Z., and J. Halouzka. 1999. West Nile Fever a reemerging mosquito-borne viral disease in Europe. Emerg. Infect. Dis. 5: 643–650.

Jupp, P. G., B. M. McIntosh, and E. M. Nevill. 1980. A survey of the mosquito and *Culicoides* faunas at two localities in the Karoo region of South Africa with some observations of bionomics. Onderstepoort. J. Vet. Res. 47: 1–6.

Kent, R. J. 2009. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol. Ecol. Res. 9: 4–18.

Latrofa, M. S., F. Montarsi, S. Ciocchetta, G. Annoscia, F. Dantas–Torres, S. Ravagnan, G. Capelli, and D. Otranto. 2012. Molecular xenomonitoring of *Dirofilaria immitis* and *Dirofilaria repens* in mosquitoes from north-eastern Italy by real-time PCR coupled with melting curve analysis. Parasit. Vectors 5: 76.

Martínez–de la Puente, J., J. Martínez, M. Ferraguti, A. Morales–de la Nuez, N. Castro, and J. Figuerola. 2012. Genetic characterization and molecular identification of the bloodmeal sources of the potential bluetongue vector *Culicoides obsoletus* in the Canary Islands, Spain. Parasit. Vectors 5: 147.

McCall, J. W., C. Genchi, L. H. Kramer, J. Guerrero, and L. Venco. 2008. Chapter 4, Heartworm disease in animals and humans. Adv. Parasitol. 66: 193–285.

Montoya–Alonso, J. A., E. Carretón, J. A. Corbera, M. C. Juste, I. Mellado, R. Morchón, and F. Simón. 2011a. Current prevalence of *Dirofilaria immitis* in dogs, cats and humans from the island of Gran Canaria, Spain. Vet. Parasitol. 176: 291–294.

Montoya–Alonso, J. A., I. Mellado, E. Carretón, E. D. Cabrera–Pedrero, R. Morchón, and F. Simón. 2011b. Canine dirofilariosis caused by *Dirofilaria immitis* is a risk factor for the human population on the island of Gran Canaria, Canary Islands, Spain. Parasitol. Res. 107: 1265–1269.

Montoya, J. A., M. Morales, O. Ferrer, J. M. Molina, and J. A. Corbera. 1998. The prevalence of *Dirofilaria immitis* in Gran Canaria, Canary Islands, Spain (1994–1996). Vet. Parasitol. 75: 221–226.

Montoya, J. A., M. Morales, M. C. Juste, A. Bañares, F. Simon, and C. Genchi. 2006. Seroprevalence of canine heartworm disease (*Dirofilaria immitis*) on Tenerife Island: an epidemiological update. Parasitol. Res. 100: 103–105.

Morchón, R., M. D. Bargues, J. M. Latorre–Estivalis, C. Pou–Barreto, R. Melero–Alcibar, M. Moreno, B. Valladares, R. Molina, J. A. Montoya–Alonso, S. Mas–Coma, and F. Simón. 2011. Molecular characterization of *Culex theileri* from Canary Islands, Spain, a potential vector of *Dirofilaria immitis.* J. Clin. Exp. Pathol. S3: 001. (doi: 10.4172/2161-0681.S3-001).

Morchón, R., E. Carretón, J. González–Miguel, and I. Mellado–Hernández. 2012. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe: new distribution. Trends Front. Physiol 3: 196.

Mukabana, W. R., W. Takken, P. Seda, G. F. Killeen, W. A. Hawley, and B.G.J. Knols. 2002. Extent of digestion affects the success of amplifying human DNA from bloodmeals of *Anopheles gambiae* (Diptera, Culicidae). Bull. Entomol. Res. 92: 233–239.

Muñoz, J., R. Eritja, M. Alcaide, T. Montalvo, R. C. Soriguer, and J. Figuerola. 2011. Host-feeding patterns of native *Culex pipiens* and invasive *Aedes albopictus* mosquitoes (Diptera: Culicidae) in urban zones from Barcelona, Spain. J. Med. Entomol. 48: 956–960.

Muñoz, J., S. Ruiz, R. C. Soriguer, M. Alcaide, D. S. Viana, D. Roiz, A. Vazquez, and J. Figuerola. 2012. Feeding patterns of potential West Nile virus transmission vectors in Southwest Spain. Plos ONE 7: e39549.

Mwangangi, J. M., C. M. Mbogo, J. G. Nzovu, J. I. Githure, G. Yan, and J. C. Beier. 2003. Blood-meal analysis for anopheline mosquitoes sampled along the Kenyan coast. J. Am. Mosq. Control Assoc. 19: 371–375.

Osório, H. C., L. Zé–Zé, and J. M. Alves. 2012. Host-feeding patterns of *Culex pipiens* and other potential mosquito vectors (Diptera: Culicidae) of West Nile virus (*Flaviviridae*) collected in Portugal. J. Med. Entomol. 49: 717–721.

Oshaghi, M. A., A. R. Chavshin, V. Hassan, Y. Fatemeh, M. Fatemeh, and N. Nahid. 2006. Effects of post-ingestion and physical conditions on PCR amplification of host blood meal DNA in mosquitoes. Exp. Parasitol. 112: 232–236.

Roiz, D., M. Roussel, J. Muñoz, S. Ruiz, R. Soriguer, and J. Figuerola. 2012a. Efficacy of mosquito traps for collection of potential West Nile mosquito vectors in a natural wetland. Am. J. Trop. Med. 86: 642–648.

Roiz, D., A. Vazquez, R. Rosà, J. Muñoz, D. Arnoldi, F. Rosso, J. Figuerola, A. Tenorio, and A. Rizzoli. 2012b. Blood meal analysis, flavivirus screening and influence of meteorological variables on the dynamics of potential mos-

quito vectors of West Nile virus in Trentino (Northern Italy). J. Vector Ecol. 37: 20–28.

Santa–Ana, M., M. Khadem, and R. Capela. 2006. Natural infection of *Culex theileri* (Diptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal. J. Med. Entomol. 43: 104–106.

Schaffner, E., G. Angel, B. Geoffroy, J.-P. Hervy, A. Rhaiem, and J. Brunhes. 2001. Lesmoustiques d'Europe. Logiciel d'identification et d'enseignement. CD ROM PC.IRD Editions.

Shaw, S. E., and M. J. Day. 2005. Arthropod-borne infectious diseases of the dog and cat. Lippincott, Williams & Wilkins, Baltimore, MD.

Simón, F., R. Morchón, J. González–Miguel, C. Marcos–Atxutegi, and M. Siles–Lucas. 2009. What is new about animal and human dirofilariosis? Trends Parasitol. 25: 404–409.

Thiemann, T. C., A. C. Brault, H. B. Ernest, and W. K. Reisen. 2012. Development of a high-throughput microsphere-based molecular assay to identify 15 common bloodmeal hosts of *Culex* mosquitoes. Mol. Ecol. Resour. 12: 238–246.

Thiemann, T. C., and W. K. Reisen. 2012. Evaluating sampling method bias in *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae) bloodmeal identification studies. J. Med. Entomol. 49: 143–149.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., and M. L. Warman. 2000. Preparation of PCR quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). BioTechniques 29: 52–54.

Ventim, R., J. Ramos, H. Osório, R. Lopes, J. Pérez-Tris, and L. Mendes. 2012. Avian malaria infections in western European mosquitoes. Parasitol. Res. 111: 637–645.

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